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Do PFAS Precursors Induce Lipid Accumulation in the Hepatocytes

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Do PFAs precursors induce lipid accumulation in hepatocytes?

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INTRODUCTION

Les substances polyfluoroalkyle et perfluoroalkyle (PFAS) constituent une classe de produits chimiques de synthèse communément utilisés dans les articles ménagers et de consommation. Ils sont trouvés dans les batteries de cuisine en Téflon ™ car ils dissuadent l'eau, la graisse et l'huile; les composés fluorochimiques se sont révélés utiles pour divers procédés de fabrication. Le sous-groupe important est constitué d'acides perfluoroalkyliques (PFAAs), qui comprennent le sulfonate de perfluorooctane (PFOS) et l'acide perfluorooctanoïque (PFOA). Chez les rongeurs et les singes, il a été démontré qu'ils augmentaient le poids du foie et provoquaient une accumulation de graisse dans le foie ou une stéatose du foie. [1–4]

PFAAs are the first generation of PFASs synthesized by companies. Little is known about other PFAS and newer shorter carbon chain length compounds that are being used to replace the original PFAS compounds since they are supposed to be better for humans and the environment. However, not enough testing has been done to prove this, and so we wanted to test these new PFAS replacements to see if they are as toxic or even more toxic than the original PFAS compound. Some of these compounds have been named precursor PFAS, because they degrade and become the old PFAAs. [1] The PFASs that we have chosen to test are perfluorooctane sulfonamide (FOSA), n-methylperfluoro-1-octanesulfonamide (MetFOSA), n-ethylperfluoro-1-octanesulfonamide (EtFOSA), 1 H, 1 H, 2 H, 2 H-perfluorooctane sulfonic acid (6:2 FTS), perfluoro-n-pentanoic acid (PFPeA (C5)), perfluoro-n-tridecanoic acid (PFTrDA (C13)), and perfluoro-n-tetradecanoic acid (PFTeDA (C14)). [5]

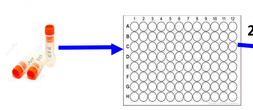
HYPOTHESIS

Based on our preliminary data and other research done, which shows that some PFAAs induce lipid deposition in hepatocytes based on chain length and functional groups, we decided that the next experiment should test PFAS precursors. Therefore, we hypothesized that the newer emerging PFASs may also have a capacity to induce lipid accumulation than legacy PFAAs. Since the experiment will be about testing the novel endpoint with emerging PFASs that are replacing widely used PFAAs, there will be three different types of procedures being used, hepatocyte treatments, nile red staining, and gene expression measurements.

MATERIALS AND METHODS

Hepatocyte plating and culture: Used a Cryostax pool of human hepatocytes from 5 donors from Xenotech LLC (Lenexa, KS). Then, cyropreserved hepatocytes were thawed and cultured on a 96-well plate following Xenotech protocols for cell suspension and counting the **dead (blue) cells** and viable **(alive) cell number (yellow)** using a hemacytometer, as well as Xenotech certified reagents such as OptiCulture Media solution and OptiMatrix.

Hepatocyte Cell Treatments:



Treatment with:
24 hrs after plating, cells were treated with PFASs at various concentrations (25 µM, 2.5 µM, and 0.25 µM) or 0.1% DMSO vehicle. Treatments were continued for **48 hrs or 72h**. Every 24 hrs, media was removed and replaced by fresh media containing treatments.

Bio-Plex analysis

Nile-red Staining

Nile-red Staining: After 72h of treatments, including lipid inducing positive control (1:2 palmitate and oleate, P/O, at 0.25 µM), cells were fixed with 10% formalin for 10 min and washed with PBS. Cells were then stained with Nile Red (3.1 µM for 10-15 min) and DAPI (300 nM for 1-5 min) and washed with PBS. Nile red fluorescence was measured (excitation 485nm/emission 535nm) was normalized to DAPI fluorescence (excitation 358nm/emission 461nm). Cells were imaged using EVOS cell imaging system.

Quantification of staining by Image J: Only the 25 µM concentrations were imaged but not including C14, in order to save time. A scale of 100 µm with a 40x magnification was used for imaging to count DAPI's nuclei and find the mean gray values of the Nile Red Images to quantify the fluorescence intensity.

Statistical Analysis from Spectrometer Instrument: Calculations were done using one-way ANOVA followed by Fisher's LSD test. All values in heat maps are average fold change values compared to DMSO controls (included on each 96-well plate), Bar graphs are means ± SEM; N = 3-4 per group

RESULTS

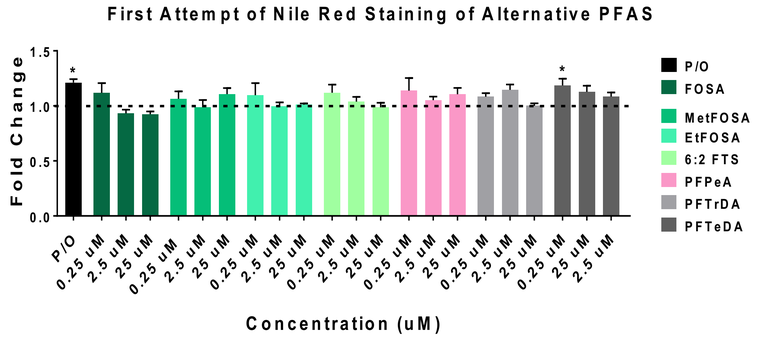


FIGURE 1: Attempt #1 Preliminary Plate 1 Data of Nile Red Staining. Neither the precursors nor the P/O fold change values were high enough to be significant , therefore the experiment was done again using the same plate design and procedures. Calculations were done using an ANOVA followed by Fisher's LSD test. All values are means ± SEM; N = 3-4. "*" indicates $p<0.05$.

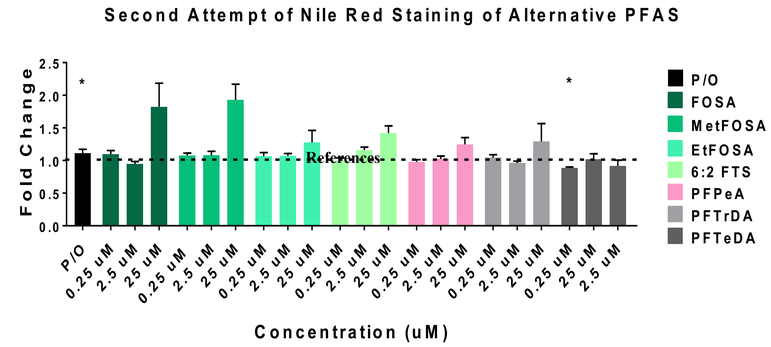


FIGURE 2: Attempt #2 Plate 1 Data of Nile Red Staining from Spectrometer Instrument Data.
Based on the Fold Change calculations, it showed that the P/O positive control was too low. Since the positive control has to be significant, meaning 1.5-2 to show an increase in lipid accumulation, the wells were looked at underneath the microscope showing that the bottom of the wells were hit too many times. So for a more accurate cell count, images were taken to quantify the fluorescence intensity.

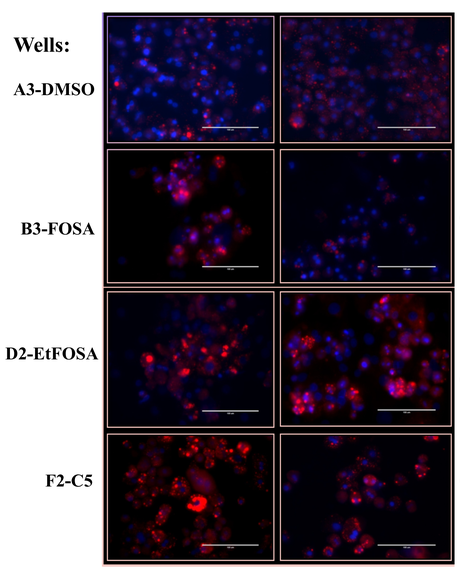


FIGURE 3: Nile Red Mean Grey Value and DAPI Nuclei Stain Images from EVOS Microscope.
These are images of Nile Red (pink/red) and DAPI (Blue) Nuclei Stains overlapping, which show that the FOSA, EtFOSA, 6:2 FTS, C5, and C13 precursors have more Nile Red staining than DAPI staining. This is the opposite for MetFOSA precursor, P/O positive Control, and DMSO. Scale=100µm. 40x magnification.

SUMMARY OF RESULTS

- Attempt #1: Preliminary data had no significance in any of the precursors nor the P/O. The fold change values were too low.
- Attempt #2: Using the spectrometer instrument showed that the numbers were still too low and that there was not much significance other than FOSA and MetFOSA precursors at the 0.25 µM concentrations.
- Image J results from Attempt #2: Shows that the P/O fold changes were not as high as 1.5 or more to be significant, so the results were not as anticipated, but it did show that FOSA and MetFOSA precursors had significant fold changes at the 25 µM concentrations.

PRELIMINARY CONCLUSIONS

Les données étant toutes similaires, il a été suggéré que l'application du traitement pourrait être problématique, mais pas le traitement lui-même. Dans les deux tentatives et l'analyse d'image, il a été connu pour augmenter l'accumulation de lipides. De plus, une des raisons pour lesquelles le nombre de cellules était si bas était due à une erreur humaine, telle que frapper le fond des puits tout en passant l'aspirateur; en conséquence, tuant certaines des cellules par accident.

FUTURE DIRECTIONS

Our next step will be to redo this experiment but with the treatment applied differently, while using spectrometer instrument analysis and quantification of nile red/DAPI staining on Image J. Then, we will conduct gene expression analysis on plate 2 using the same plate design, except P/O will be replaced with rosiglitazone (ROSI) and next to it in the 4 wells, we will add clofibrate (CLO).

REFERENCES

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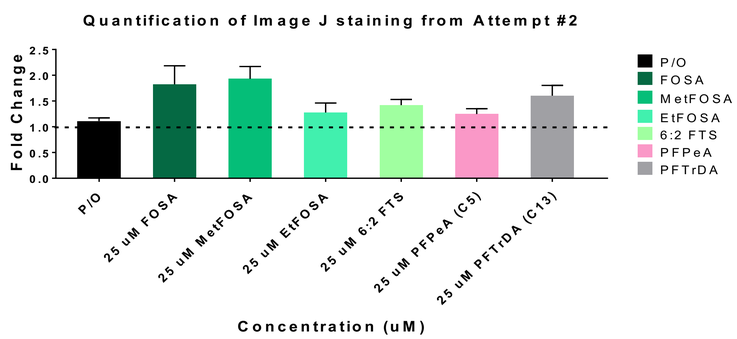

FIGURE 4: Quantification of Image J staining from Attempt #2 of Plate 1.
Human hepatocytes were treated with PFAS precursors for 48 hours and stained with nile red and DAPI. Staining for the 25 µM treatment groups was quantified by Image J for each of the wells. Only the 25 µM concentrations were imaged not including C14, in order to save time. FOSA and MetFOSA increased the hepatic lipid staining.